

Perspective

# On Catalytic Kinetics of Enzymes

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**Abstract:** Classical enzyme kinetic theories are summarized and linked with modern discoveries here. The sequential catalytic events along time axis by enzyme are analyzed at the molecular level, and by using master equations, this writing tries to connect the microscopic molecular behavior of enzyme to kinetic data (like velocity and catalytic coefficient  $k$ ) obtained in experiment:  $1/k = t$  equals to the sum of the times taken by the constituent individual steps. The relationships between catalytic coefficient  $k$ , catalytic rate or velocity, the amount of time taken by each step and physical or biochemical conditions of the system are discussed, and the perspective and hypothetic equations proposed here regarding diffusion, conformational change, chemical conversion, product release steps and the whole catalytic cycle provide an interpretation of previous experimental observations and can be testified by future experiments.

**Keywords:** catalysis; kinetics; time; enzyme; macromolecule; biological macro-substrate; catalytic step; catalytic efficiency; catalytic coefficient



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## 1. Introduction

Some of the basic theories of biochemistry come from chemistry [1–3], which deals with small molecules most of the time. Although enzyme kinetic principles transplanted from chemistry have changed a lot to adapt to biological specificity [3], some of the description can be improved to better reveal the nature of molecular events of biochemical catalysis. This is important for the advancement of not only science, but also biochemical engineering, drug discovery and other applications or technologies as well. In fact, enzyme kinetics is correlated with the time course of sequential catalytic events.

Catalytic rate/velocity depends on the amount of time the enzyme takes to successfully convert a certain amount of substrate molecules into products [4,5]. The catalytic process actually includes not only the traditionally defined chemical-transformation step (which may include multiple sub-steps itself), but also other related physical, biophysical or biochemical catalytic steps, for instance, diffusion, enzyme–substrate recognition or binding, conformational change and product-release steps [6]. This is the case for chemical reactions as well as biochemical reactions catalyzed by enzymes. If any of these “trivial” steps take time to accomplish, it will affect the overall catalytic rate and cannot be ignored if accuracy is required. Here, this writing tries to discuss the complete catalytic cycle as a whole. Turnover, catalytic step, enzyme, catalysis and catalytic cycle in the following discussion mean to refer to those related to biochemical reactions in homogeneous stable aqueous solution catalyzed by enzymes, unless stated otherwise. This writing may be applied to other systems, reactions or catalysis as well.

To start with, time scales of fundamental steps of a catalysis will be discussed from the molecular model point of view. Protein with a molecular weight of 64 kDa diffuses at

a rate of about  $5 \mu\text{m}\cdot\text{s}^{-1}$  in cells [7]. It takes roughly  $10^{-7}$  s on average for one molecule to meet with another in aqueous solution, with a concentration of 10 mM, and if the concentration is 1  $\mu\text{M}$ , the time is  $\sim 10^{-3}$  s [8,9]. Tumbling of proteins in aqueous solution is at nanosecond ( $10^{-9}$  s) time range [10]. Local motions of an enzyme, like the motions of side chains of surface residues, take roughly  $10^{-12}$ – $10^{-9}$  s; it takes longer when intramolecular residues or residues with large bulky side chains are involved [11]. Medium-scale conformational change up to several Angstroms like loop motion, hinge bending motion and some domain movement takes usually about  $10^{-9}$ – $10^{-4}$  s to accomplish [12–15]. Large-scale conformational change takes about  $10^{-4}$ – $10^0$  s to accomplish [16], and some large-scale conformational change can take an even longer time. The further the movement and the larger the moving portion, the longer time it will take. The amount of time taken for the substrate-to-product chemical conversion step by different enzymes varies a lot, from  $10^{-7}$ – $10^0$  s to a considerably long time [17,18]. From these listed numbers, it is obvious that diffusion process, reorientation, recognition/tethering and the conformational change step can happen in similar time scales as the substrate-to-product chemical conversion step [8–20], thus possibly affecting the catalytic rate as such.

Besides the chemical step, other steps can be rate limiting as well, both in theory and in reality. Turnover number  $k_{\text{cat}}$  of the  $\text{H}_2\text{O}_2$  to water plus dioxygen reaction catalyzed by catalase is around  $4 \times 10^7 \text{ s}^{-1}$  [17]. This value means that the catalytic rate in certain circumstances can be partially limited by diffusion as well. In several other cases, binding, conformational change or product release are the rate limiting steps, respectively, and these facts have been supported by numerous experiments by different technologies [21–31]. Enzyme catalyzed reactions have a lot of steps involved; theoretically, any step can be rate limiting. All the catalytic steps contribute to the catalytic efficiency.

## 2. The First Special Assumption and General Assumption

All the reactions catalyzed by any free enzymes in homogenous stable aqueous solution systems should follow the same unified general principle; there should be no exceptions. This is the first assumption, which leads to master equations. The special assumption specifically concerns homogenous stable aqueous solution systems for a certain period of time during which variables of the catalysis (like the substrate concentration or catalytic velocity) are relatively constant, while the general first assumption does not confine the system nor the catalysis.

“Homogeneous stable aqueous solution system” means that all the participants of the catalysis are homogeneously distributed within the solution system and are freely diffusible in the stable fixed-volume solution. There shall be no denaturants in the system, so that the enzyme is properly folded and active. All the way through the catalytic process, such mild relatively constant conditions are maintained. If the system goes so far away from normal physiological conditions that the enzyme gets denatured, the discussion here may not stand valid for the case anymore.

If membrane protein is solubilized by detergents (or lipids) and is freely diffusible and a homogeneous stable aqueous solution system is also formed, both the substrate and the product are water soluble, and the catalytic center locates at the solvent exposing surface and everything behaves very much like a water-soluble enzyme and substrate in aqueous solution system, then this is still within the scope of the first assumption.

Membrane integrated enzymes restrained in two-dimensional lipid bilayer systems are different from soluble enzymes. In the first circumstance, membrane-protein enzyme is in lipid-bilayer systems with the catalytic center exposed to the solvent, and both the substrate and product are water soluble. It is like only the enzyme is floating within the two-dimensional space; the diffusion process/kinetics are special in that only substrate and product diffuse freely in aqueous solution. Conformational change kinetics of enzymes can be unusual in that lipid molecules are involved as well. In the second circumstance, both the membrane-protein enzyme and the substrate are hydrophobic and restrained in lipid-bilayer systems; the diffusion of both enzyme and substrate in lipid bilayer is

constrained in this two-dimensional space [32]. The diffusion and the conformational change manners are distinct from those in aqueous solution.

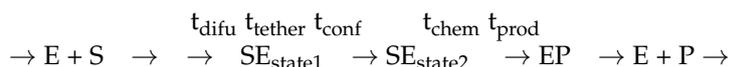
Presumptions of previous kinetic theories include steady-state assumption, transient-state assumption, equilibrium/quasi-equilibrium assumption etc. Because of the presence of exceptions [3,4,33,34], none can be the universal basic presumption of catalysis. Another basic issue is about rate limiting factors. Scientists used to believe that there were two distinct kinds of reactions in solutions, diffusion-controlled reaction [9,35,36] and activation-controlled reaction [1,37,38]; the rate constants were also expressed in distinct equations. Activation energy is required for the chemical-conversion step [1]; if the chemical conversion is so fast that the diffusion step becomes rate limiting, then it is a diffusion-controlled reaction [9,35,36]. These two cases reveal two important common rate-limiting sources or origins in solution. Actually, as discussed above, other steps like binding or conformational change have been shown nowadays sometimes to be rate limiting as well.

The third issue to be discussed is about the relative amount of reactants. For steady-state approximation, the number of substrate molecules needs to be much greater than the number of enzyme molecules. The classical definition of turnover number ( $k_{\text{cat}}$ ) is preserved here: "It is equivalent to the number of substrate molecules converted to product in a given unit of time on a single enzyme molecule when the enzyme is saturated with substrate." But the concentration of enzyme is not always so negligible as supposed. Therefore, a different more general parameter  $k$  as catalytic coefficient (catalytic cycles per unit time per single enzyme in like  $\text{s}^{-1}$ ) to reveal the catalytic efficiency of enzymes at various substrate concentrations is defined here. The concentration of large biological macro-substrates (LBMS) is usually lower than that of small-sized low-molecular-weight substrate (SMS) in the cell [39–41]; the enzyme catalytic center is readily accessible to the SMS. LBMS diffuses slower and rotates slower than SMS, which all make LBMS take additional and longer time to diffuse, to meet with the enzyme and to take the right orientation and accommodate specific parts into the catalytic pocket, both in vivo and in vitro. Much more importantly, it is always a mutual process of recognition between the enzyme and LBMS [26,28,42,43]. In this case, the time taken by binding/tethering/recognition and conformational change process, etc., can no longer be neglected; it has to be taken into consideration. In other words, the experimentally obtained catalytic velocity or rate actually includes the contributions of all steps (including sometimes significant contributions from other steps like conformational change and product release besides the chemical-conversion or diffusion step), no matter if the researcher realizes it or not. This is the case for both LBMS and SMS, and for the reasons discussed above, it is more important and serious for LBMS. For physiologically related enzyme catalysis, the concentration of LBMS is usually at similar order of magnitude with that of the enzyme [41]. The presumption that the concentration of the enzyme is negligible if compared to that of the substrate will not typically stand true anymore for LBMS in vivo. Therefore, it is quite a different scenario for LBMS involved catalysis. If the concentrations of both enzyme and substrate are taken into consideration, the description about the catalysis will be more reasonable.

As discussed above, enzyme catalyzed reactions are so diverse that these issues are fundamentally distinct for different cases, and cannot be the universal basic presumption of catalysis. Enzyme kinetic theories, including Michaelis–Menten Kinetics, Briggs–Haldane's theory, Quadratic Velocity Equation (tight-binding equation or the Morrison equation) and those theories on enzymatic rate enhancement, etc., play important roles in the research of catalysis and enzymology [1,5,44,45]. Aside from these principles, some other universally suitable kinetic principles may be extracted from innumerable available examples now.

The unidirectional irreversible continuous flow of time is commonly regarded as invariant in the universe, therefore, with the first general presumption for any one enzyme molecule that catalysis proceeds step by step following sequential course or chronological order, this writing uses the master equations based on the sequential events along the time axis to study the kinetics of enzyme catalysis (Catalytic scheme 1). In a certain time window of homogenous stable aqueous solution systems, master equations are applicable at both

microscopic and macroscopic levels, and this expresses the first special assumption. For the many time windows of homogenous stable aqueous solution systems, the first special assumption may be valid for each one individually. For circumstances like catalysis in flow of multiphase mixtures, catalytic process with immobilized participants, or catalysis in systems with changing/unstable conditions (changing temperature, pressure, viscosity or chemical composition etc.), the perspective here, although it can be helpful, may not be applied directly for the investigation. For inhomogeneous or changing systems, the master equation can be applied directly at microscopic level to a single individual catalytic cycle, but at macroscopic level, only after sophisticated dissection into both different time windows and various 3D space sections or volumes can statistical averaging be possible.



Catalytic scheme 1. Representative steps of catalysis are shown here. Reversible reaction of any step may occur, which is not excluded by this scheme. The single-headed arrow means to highlight only that at microscopic level,  $t_i$  refers to the time actually taken by the successful accomplishment of step  $i$ , and at macroscopic level,  $t_i$  refers to the statistically averaged time of enzyme ensembles taken by step  $i$ . The time taken by reverse reaction is not shown in the scheme because it has already been statistically averaged (included) into time  $t_i$ . The arrows at the very left and very right mean to express that this turnover may not be an isolated catalytic cycle; the enzyme may be from a previous catalytic cycle and may enter another one.

Under the first special assumption for constant catalysis in homogenous stable aqueous solution systems, master equations are valid both microscopically and macroscopically; while under the first general assumption, master equations are directly applicable surely only at microscopic level.

### 3. Master Equations

Enzyme catalysis is analyzed from two interconnected point of views, macroscopic and microscopic. When the overall enzyme ensembles in a given system are concerned, the catalytic event of a statistically-averaged representative or virtual single turnover by a single enzyme will be highlighted to explain what is happening to the molecules in a batch. The kinetic experiment obtained parameters like velocity/rate and catalytic coefficient  $k$  are actually statistics-, distribution- and frequency-averaged values of enzyme ensembles, rather than the behavior of a single individual enzyme molecule, nor any component step. A single catalytic cycle of a single individual enzyme may be stochastic, random and be affected by a lot of occasional factors. The distribution of single catalytic cycles at both space and time dimensions is something that really existed and shall be studiable. On the other hand, if any single catalytic cycle is specifically concerned, like the very first one, it is obvious that this writing and the master equations discussed below will be readily applicable directly. Singular-enzyme behavior obtained by single-molecular technologies is statistically linked with bulk behavior of enzyme ensembles (like the catalytic coefficient  $k$  and catalytic rate/velocity obtained from the kinetic experiment) as well.

Consider the whole picture of a single turnover (or single catalytic cycle) of an enzyme catalyzed multiple-turnover reaction, the enzyme and the substrate have to firstly diffuse to meet with each other; the reactants need to rotate to the right orientation to tether, to recognize and to bind, and the enzyme performs conformational change; then the substrate is converted to product through the chemical-conversion step; and then the product is released and the enzyme enters another catalytic cycle. With all the sequential catalytic events in chronological order, the whole catalytic cycle is like a pipeline; although there may be bottle necks, each and every component step, if the catalytic cycle can be divided into discrete elementary steps, takes time to accomplish and contributes to (or limits) the catalytic efficiency and velocity. Actually, as the biochemical catalysis proceeds, each cycle of it will have to get through every single constituent step and cannot skip any one.

The amount of time that one turnover takes stems from the combination of each single step of the catalytic cycle. All of the time-consuming steps limit the overall catalytic efficiency of catalysis. If any step takes such short time on average that it is negligible in comparison with other steps, then it can be omitted for simplicity, and which step to ignore depends on the situation. These diffusion, tethering or recognition, conformational change, chemical or biochemical conversion and product release processes happen in chronological order, and recent studies have shown that elementary steps of catalysis are independent of and separable from each other [23,30,31]. Let the coherent process be carefully divided so that each step simply does not overlap in time axis with one another, then the amount of time taken by these sequential steps become addable. Effectual diffusion is an independent step without overlap in time course with other steps, in the same catalytic cycle or from nearby cycle; in very viscous systems or cases of LBMS or diluted reactants, diffusion takes a considerable amount of time.

The catalytic process in a system is like this, many enzyme molecules are working in parallel: each one conducts tandem repeats of catalytic cycles, and the enzyme molecules are usually not synchronized. Normally the enzyme will visit each of every unit step periodically. As for the starting point of a single turnover of the many continuous catalytic cycles, it is up to the situation. Although for the many different enzyme molecules in a system the amount of time each takes to accomplish a single turnover may be different, the amount of time may distribute in a certain manner. Although for even the same enzyme molecule conducting multiple catalytic cycles, the time span of each turnover may be different, which may follow a certain distribution. Although the amount of time taken by any one specific step of the many sequential steps of many turnovers by the many enzyme molecules may vary from one catalytic cycle to another, which may follow a certain distribution. Let the statistically averaged representative single catalytic cycle by a single enzyme be analyzed, all other enzyme molecules will be copies of this one; let time flow and catalytic cycles will be periodical tandem repeats of this single catalytic cycle.

Let there be  $n$  steps within a single turnover in an enzyme catalyzed multiple turnover reaction; within a single turnover, each step  $i$  takes time  $t_i$  to accomplish. Then the total amount of time  $t$  taken by a whole single turnover is the sum of the time taken by all the steps.

$$t = \sum_{i=1}^n (t_i) \quad (1)$$

Both  $t$  and  $t_i$  have real biophysical meanings, at the microscopic single molecular level,  $t$  is defined as the total time taken by a typical single turnover of a single enzyme and  $t_i$  is the amount of time taken by step  $i$  within the typical single turnover, both may be obtainable by single molecular technologies. At macroscopic level,  $t$  and  $t_i$  are actually statistically averaged amounts of time of enzyme ensembles taken by a catalytic cycle and specific step, respectively. Catalytic coefficient  $k$  is equivalent to the number of substrate molecules successfully converted to product per unit time by a single enzyme molecule (or per single enzyme active site), not necessarily at substrate-saturation condition. If catalytic coefficient  $k$  is obtained at substrate saturation condition, then  $k = k_{\text{cat}}$ . Then,

$$kt = 1 \quad (2)$$

$$k \cdot (t_1 + t_2 + t_3 + \dots + t_i + \dots + t_n) = 1 \quad (3)$$

These three equations are the frame of this writing. At microscopic level,  $k$  stands for catalytic cycles achieved by a single enzyme within one-unit time, which may be obtainable by single molecular technologies; at macroscopic level,  $k$  is the averaged catalytic efficiency of enzyme ensemble (velocity divided by enzyme concentration,  $V/[E]$ ), which can be obtained by kinetic experiment. And catalytic coefficient  $k_i$  of step  $i$  is defined as the catalytic-cycle number per unit time of a step- $i$ -dedicated single enzyme. Similar to  $k$ ,  $t$  and

$t_i$ ,  $k_i$  can be viewed at both the microscopic and macroscopic levels; for simplicity, similar explanations will not be repeated.

$$t_i k_i = 1$$

The catalytic coefficient  $k_i$  is the largest achievable catalytic-cycle number of step  $i$  (fastest possible velocity of step  $i$   $V_i$  over respective molecule or complex concentration). Imagine the enzyme is devoted to step  $i$  and doing nothing else, and substrate of step  $i$  or product of step  $i-1$  is immediately available in excess. Then,  $k_i$  of any step  $i$  will be larger in value than  $k$ ; this means if the enzyme catalyzes only that single step, it will result in more turnover cycles. Because normally the enzyme is busy with other catalytic steps during time  $t-t_i$ , the overall output of the whole process (or within the whole time  $t$ ) will decrease to a level below the throughput capacity (or flux) of any single step  $i$  of the catalytic cycle.

If any one step  $i$  is the only rate-limiting step, and  $t \approx t_i$ , then  $k \approx k_i$ . This step  $i$  can be the diffusion step (a diffusion-controlled reaction), or the enzyme conformational change step, or the substrate-to-product chemical conversion step (an activation-controlled reaction), or the product-release step, or some other step. There are times when the second most time-consuming step  $j$  also takes a considerable amount of time, for instance,  $t_j/t > 20\%$ . Then the two steps  $i$  and  $j$  are both rate limiting; the throughput capacity of other steps are so big that they are all "waiting" for these two steps; the  $t - t_i - t_j \approx 0$ , then  $k \approx k_i k_j / (k_i + k_j)$ . There are cases when the third most time-consuming step  $x$  also takes a considerable amount of time, for instance,  $t_x/t > 10\%$ , the  $t - t_i - t_j - t_x \approx 0$ , then  $k \approx k_i k_j k_x / (k_i k_x + k_j k_x + k_i k_j)$ . Similar equations can also be deduced, and so on.

For simplicity, five major steps will be discussed here, namely, the diffusion step, the tethering step, the reactant conformational change step, the substrate-to-product chemical conversion step and the product-release step. Each of the five steps takes time  $t_{\text{difu}}$ ,  $t_{\text{tether}}$ ,  $t_{\text{conf}}$ ,  $t_{\text{chem}}$  and  $t_{\text{prod}}$  on average within a single turnover, respectively. For a certain catalysis, if all other steps can be ignored, then for the whole single turnover, time  $t \approx t_{\text{difu}} + t_{\text{tether}} + t_{\text{conf}} + t_{\text{chem}} + t_{\text{prod}}$ . Catalytic coefficient within one-unit time  $k = 1/t$ , and  $k_{\text{difu}} = 1/t_{\text{difu}}$ ,  $k_{\text{tether}} = 1/t_{\text{tether}}$ ,  $k_{\text{conf}} = 1/t_{\text{conf}}$ ,  $k_{\text{chem}} = 1/t_{\text{chem}}$ ,  $k_{\text{prod}} = 1/t_{\text{prod}}$ , then

$$k \approx k_{\text{difu}} k_{\text{conf}} k_{\text{chem}} k_{\text{prod}} k_{\text{tether}} / (k_{\text{difu}} k_{\text{conf}} k_{\text{prod}} k_{\text{tether}} + k_{\text{conf}} k_{\text{chem}} k_{\text{prod}} k_{\text{tether}} + k_{\text{difu}} k_{\text{chem}} k_{\text{prod}} k_{\text{tether}} + k_{\text{difu}} k_{\text{conf}} k_{\text{chem}} k_{\text{tether}} + k_{\text{difu}} k_{\text{conf}} k_{\text{chem}} k_{\text{prod}})$$

The five major steps are present in all biological catalysis, although for some enzymes or catalysis, one or more of these steps take time that is negligible; for some others, there may be additional major steps involved.

$t_{\text{difu}}$ , diffusion time, is the time taken on average for an effective enzyme–substrate encounter within the aqueous solution system. After diffusion, the reactants are physically close to each other; the substrate may rotate, roll, crawl or hop on the surface of the enzyme for a successful in-catalytic-pocket binding. The recognition and tethering process is dependent on the surface property of the enzyme and the substrate, like electrostatic property, the shape and hydrophobicity, etc. Tethering along with solvation/desolvation is an important and sophisticated process distinct from and closely related to diffusion. Tethering can be distinct from conformational change, but sometimes tethering or binding process itself means large conformational change, and only a limited amount of pioneering experimental results are available for a systematic recapitulation [19,46].

$t_{\text{chem}}$  is the total time spent on average by the chemical-conversion step within a single turnover of a catalysis.  $t_{\text{chem}}$  is independent of free substrate concentration or free enzyme concentration. For this chemical-conversion part of a catalytic cycle, many classical biochemical principles still apply, like the transition state theory and Arrhenius equation, etc. For multistep chemical conversions, there can be  $t_{\text{chem}1}$ ,  $t_{\text{chem}2}$ ,  $\dots$ , and  $t_{\text{chem}} = t_{\text{chem}1} + t_{\text{chem}2} + \dots$ .

$t_{\text{conf}}$  is the amount of time taken by conformational change of reactants within a single catalytic cycle;  $t_{\text{conf}}$  is a parameter dependent mainly on the molecules' intrinsic structure and character, forces from other macromolecules and the environment like temperature

of the system, etc. Sometimes, the enzyme–substrate complex (or enzyme–substrate–modulator complex) performs conformational change as a whole.  $t_{\text{prod}}$  is the amount of time taken on average by the product-release step within a single catalytic cycle.

$t_{\text{phys}}$  is the total time taken by the physical or biophysical steps within a single turnover of a specific catalysis, including the time required for enzyme and substrate diffusion, rotation, tethering/binding, reactant conformational change and product-release process, etc. Although protonation/deprotonation can also take some time and is neither always part of the substrate-to-product chemical conversion process nor always the biophysical process, it is usually short and too complex and diverse to be scrutinized here. In one word,  $t_{\text{phys}}$  is the sum-time of all the physical steps before and after the chemical conversion;  $t_{\text{phys}}$  is the preparation time for chemical conversions to occur. If only diffusion, tethering, reactant conformational change and product-release steps account for the majority of physical process, then  $t_{\text{phys}} \approx t_{\text{difu}} + t_{\text{tether}} + t_{\text{conf}} + t_{\text{prod}}$ . The chemical-conversion step is quite different from physical or biophysical steps in at least the following aspects, ① strong-covalent-bond change, ② driving forces, ③ rate limiting origins and ④ activation energy involved or not.

The master equations indicate at least the following.

- ① The step which is the most time-consuming will be the primary “rate-limiting” or efficiency-limiting step. Traditionally, the step which requires the most activation energy is regarded as the rate-limiting step. This may still stand correct for the sub-steps of the substrate-to-product chemical conversion step, but it cannot be applied beyond. Although the “rate-limiting step” can be the one to optimize to greatly improve the overall catalytic efficiency and catalytic rate, it is not the sole factor that dictates the catalytic efficiency, but all the steps combined together.
- ② The amount of time taken by each step of a catalytic cycle is addable and the above discussion has explained how. But catalytic velocity (concentration per unit time measured in like  $\mu\text{M}\cdot\text{min}^{-1}$  or  $\text{mM}\cdot\text{s}^{-1}$ ) of each step or catalytic coefficient of each step (e.g.,  $k_i$ ,  $k_j$  in like  $\text{s}^{-1}$ ) are not directly addable. For easy comprehension, this writing will definitely be directly applicable to unidirectional irreversible catalysis; the general concept here shall be applicable for studying other catalysis as well. In this writing, the velocity of each step is the throughput capacity of the step within the fixed-volume stable system, and velocity of the whole catalytic cycle is the net velocity.
- ③ The experimentally obtained catalytic rate/velocity divided by enzyme concentration actually equals to the  $k$  from the master equation discussed above, rather than the catalytic coefficient  $k_i$  of any single step  $i$ .  $k=k_{\text{exp}} = V_{\text{exp}}/[E_t]$ ,  $[E_t]$  is the total committed active enzyme concentration and  $V_{\text{exp}}$  is the experimentally obtained catalytic velocity. Kinetic experiment obtained single-turnover time  $t_{\text{exp}} (=1/k_{\text{exp}})$  is the average of many turnovers catalyzed by the enzyme ensemble of the system. It also equals to the sum of the time actually spent by all the steps within a certain representative single catalytic cycle.

$$[E_t]/V_{\text{exp}} = 1/k_{\text{exp}} = t_{\text{exp}} = \sum_{i=1}^n (t_i)$$

Again, if only diffusion, tethering, conformational change, chemical conversion and product-release steps account for the majority of catalytic time,  $1/k_{\text{exp}} = t_{\text{exp}} \approx t_{\text{difu}} + t_{\text{tether}} + t_{\text{conf}} + t_{\text{chem}} + t_{\text{prod}}$ .

The master equations here provide a framework for the analysis of catalytic kinetics of enzymes, relationships between catalytic efficiency, catalytic rate/velocity, the amount of time taken by each step and physical/biochemical conditions of the system at diffusion, conformational change, chemical conversion, product-release steps and for the whole catalytic cycle will be discussed at each individual section, and all the essential steps are linked together by master equations.

#### 4. The Second Assumption

To postulate the correct equation to describe something comprehensively and insightfully, intrinsically independent parameters need to be defined first, and only in this way will the conclusions obtained be useful and applicable to practices. Otherwise, the contributions of multiple factors cannot be distinguished from one another and the question under investigation will not be dissected meaningfully.

Overall catalytic rate/velocity ( $V_{\text{overall}}$ ) is correlated with reactant concentration, the properties of the enzyme and the physical and chemical conditions of the system, etc. Relatively independent factors that potentially affect  $V_{\text{overall}}$  need to be segregated and identified. The catalytic coefficients  $k_{\text{chem}}$ ,  $k_{\text{conf}}$ ,  $k_{\text{prod}}$  and  $k_{1-\text{difu}} = 1/(t_{\text{difu}})$  of any enzyme are parameters that correlate with and only with the intrinsic characters of the enzyme (or the enzyme–substrate complex, etc.), the temperature  $T$ , the pressure  $P$ , the viscosity  $\eta$ , the density  $\rho$  and other biophysical/biochemical properties of the system. Except  $k_{\text{difu}}$  and thus  $k$ , the catalytic coefficient of all other steps is totally independent of free substrate concentration. This is the second assumption.

Intrinsic characters of the enzyme that potentially affect the activity of the enzyme used in the experiment include all those factors, like the primary sequence, three-dimensional structure or conformation, the modification state of the enzyme, whether the enzyme is apo or holo (with cofactors incorporated or not), with modulators or effectors or inhibitor or activator bound or not, the presence or absence of other attached regulatory molecules, etc. [24,47–49].

The properties of the solution system include physical (like temperature, pressure, viscosity, density, etc.) [50–52] and chemical conditions. The latter includes the pH, the ion strength, types and concentration of solute or electrolyte, the presence and concentration of certain chemicals or loose interactors like effectors, regulators, substrate analogues, etc. The most suitable chemical condition for catalysis is different from enzyme to enzyme. Chemical conditions will affect the catalysis or more specifically the catalytic coefficient differently from case to case.

If we'd like a catalytic coefficient to reveal the properties of the enzyme and the physical and chemical conditions of the system, like the pressure, the temperature, the viscosity, density, etc., then it should have nothing to do with substrate concentration, enzyme concentration, or enzyme–substrate complex concentration. The catalytic coefficients  $k_{\text{chem}}$ ,  $k_{\text{conf}}$  and  $k_{\text{prod}}$  are parameters of this kind. With contributions of physical conditions of the system to these catalytic coefficient parameters clearly defined, ultimately, factors/constants representing the properties of the enzyme at specific chemical conditions will be obtained, which is useful for application research and practices.

#### 5. The Third Assumption

The  $k_{\text{difu}}$ , collision or encounter rate  $V_{\text{difu}}$  ( $V_{\text{difu}} = k_{\text{collision}} [E][S]$ ), thus the overall catalytic coefficient  $k$  and overall catalytic rate/velocity  $V_{\text{overall}}$ , should be correlated with and depend on free substrate concentration  $[S]$  [9,35,36]; velocity is measured in concentration per unit time (in like  $\text{Ms}^{-1}$ ,  $\text{mol}\cdot\text{L}^{-1}\cdot\text{s}^{-1}$ ). The rate or velocity of chemical-conversion step depends on enzyme–substrate complex concentration  $[ES]$ ,  $V_{\text{chem}} = k_{\text{chem}} [ES]$  or  $V_{\text{chem}} = k_{\text{chem}} \cdot [ES_1S_2S_3\cdots]$ . If conformational change of ES complex accounts for the major part of conformational change step, then the rate or velocity of conformational change step depends on enzyme–substrate complex concentration  $[ES]$ ,  $V_{\text{conf}} = k_{\text{conf}} [ES]$  or  $V_{\text{conf}} = k_{\text{conf}} \cdot [ES_1S_2S_3\cdots]$ . This is the third assumption.

Velocities of conformational change, chemical conversion or product-release steps are not directly linked with free substrate concentration. The Michaelis–Menten equation describes and only describes the relationship between initial velocity  $V_0$  (concentration per unit time in like  $\mu\text{M}\cdot\text{min}^{-1}$  or  $\text{mM}\cdot\text{s}^{-1}$ ) and substrate concentration [45,53], and it is appropriate for the steady state [3] (or rapid equilibrium [33] or reactant stationary assumption/condition [54]) initial velocity analysis when the product is generated at a linear velocity and the catalytic rate shows linear dependence on active enzyme concentration  $[E]$ .

What does  $K_m$  mean down to the bottom?  $K_m/V_{max}$  is the linear dependence index of  $1/V_0$  on  $1/[S]$ . For catalysis not obeying Michaelis–Menten kinetics, there can be alternative equations describing the relationship between  $V_0$  and  $[S]$ .

An experimentally obtained possible relationship between substrate concentration and initial velocity is like this [33] (Figure S1). With the increase of substrate concentration  $[S]$ , the initial velocity  $V_0$  is ever growing until it reaches a plateau near  $V_{max}$  (concentration per unit time), as the substrate concentration gets to near saturation. The Michaelis–Menten equation well describes the shape of the curve.

As an equation summarized from experience, if the Michaelis–Menten equation is applied at different substrate concentration  $[S]$  windows, the obtained parameter  $V_{max}/K_m$  will bear distinct innate meanings. When  $[S]$  is small,  $k_{collision} \cdot [S] \cdot [E]$  is small and the diffusion step is rate limiting. Within certain low  $[S]$  ranges (the left bottom corner of the curve), the Michaelis–Menten equation can be used to obtain the linear dependence index  $K_m/V_{max}$  of  $1/V_0$  on  $1/[S]$ . As an approximate diffusion term,  $V_{max}/K_m$  now has real biophysical definitions. As  $[S]$  increases to the middle part of the curve, maybe both diffusion and other steps like chemical-conversion step are rate limiting. When  $[S]$  is near saturation, steps like chemical conversion other than diffusion are the major rate-limiting steps. Even with the further increase of  $[S]$ ,  $t_{diffu}$  will not become noticeably shorter and diffusion will not improve the overall throughput of the catalysis significantly. As discussed before,  $[S]$  will not directly affect the values of  $t_{conf}$ ,  $t_{chem}$ ,  $t_{prod}$ ,  $V_{conf}$ ,  $V_{chem}$  or  $V_{prod}$  either. When  $[S]$  increases to the point where steps like chemical conversion (or conformational change, steps other than the diffusion step) start to be involved in rate-limiting, the obtained “dependence index”  $K_m/V_{max}$  of  $1/V_0$  on  $1/[S]$  becomes a parameter with mixed contributions from both the diffusion step and other steps like the chemical conversion step, and the researcher simply cannot tell how much each one contributes, unless additional examination is carried out. Then the obtained  $V_{max}/K_m$  loses its original denotation and is no longer an approximate diffusion term. A direct relationship between catalytic coefficient ( $k_{conf}$ ,  $k_{chem}$ ,  $k_{prod}$ ) of conformational change, chemical conversion or product-release step and free substrate concentration  $[S]$  is pointless. Therefore, the same parameter “dependence index”  $K_m/V_{max}$ , if obtained at different  $[S]$  regions, conveys totally different information.

Then comes another question: When  $[S]$  gets to saturation, is the experimentally obtained turnover number  $k_{exp-cat}$  standing for that of the chemical conversion step? Not really. Is there any direct relationship between this  $k_{exp-cat}$  and the activation energy  $E_a$ ? No necessary direct correlation. Activation energy  $E_a$  is only correlated with the chemical-conversion step. When  $[S]$  gets to saturation, we can merely say that only diffusion time  $t_{diffu}$  is definitely negligible, which means that  $1/k_{exp-cat} \approx t_{conf} + t_{tether} + t_{chem1} + t_{chem2} + t_{prod}$ , if other steps are negligible as well. From the curve, it is obvious that, like any other trivial steps, the amount of time spent by diffusion is always there, with the increase of  $[S]$  it can be ignored, but it never really disappears.

The catalytic coefficient (in like  $s^{-1}$ ), rate or velocity (concentration per unit time in like  $\mu M \cdot \text{min}^{-1}$  or  $mM \cdot s^{-1}$ ) and extent of catalysis per unit time  $d\xi/dt$ , defined as the quantity of substrate molecules converted to product per unit time by all the committed active enzyme molecules in the system (measured in amount per unit time in unit like  $\text{mol} \cdot s^{-1}$ ), have something in common in essence: they all indicate the throughput of the catalysis in a given unit of time, although they are represented in different ways. Kinetic experiment obtained values of these parameters are actually net values, and they are linked with the equations discussed here.

## 6. Diffusion Process

The diffusion step is a process in which the enzyme and the substrate diffuse in aqueous solution to reach each other. Brownian motions of substrate and enzyme take place and contribute to the homogeneous distribution of the system. The diffusion process is different from other steps in that usually at least two free participants are involved, and

one complex is formed after tethering. Diffusional-movement velocity of molecule depends on the molecular weight, viscosity, temperature and density of the system, etc. [55–59].

An enzyme catalyzed reaction will only occur if the reactant molecules/particles come within a distance  $R^*$  from each other. Then the rate of the encounter will be dependent on the frequency of molecular collisions [9,35,36]. Problems of complex diffusion process of multiple reactants can usually be dissected into the diffusion and collision of two, e.g., first between reactant1 and reactant2, then between reactant1-2 complex and reactant3, etc. Here, the case of one enzyme and one substrate will be taken as an example for the following (and above) discussion. In addition, for example, an enzyme catalyzes the modification of a LBMS using compound1 in the presence of ATP. Although multiple routes are possible, the formation of enzyme-LBMS-compound1-ATP quaternary complex via diffusion can usually be roughly estimated through the investigation of diffusion and encounter of enzyme and LBMS.

In the first circumstance, there is no distant attraction or repulsion between the enzyme and substrate; the two reactants only come to each other by chance. According to Einstein and Marian Smoluchowski, in aqueous solution,

$$\text{Collision/encounter rate} = 4\pi R^* (D_E + D_S) N_A \cdot [E] \cdot [S]$$

$$\text{Collision rate constant } k_{\text{collision}} = 4\pi R^* N_A (D_E + D_S),$$

$$k_{\text{collision}} = 4\pi R^* N_A [k_B T / (c_E \pi \eta R_E) + k_B T / (c_S \pi \eta R_S)],$$

$\pi$  is a constant with a value  $\sim 3.14159265$ ,  $D_E$  and  $D_S$  are the diffusion coefficients of the two reactants (enzyme and substrate) in solution,  $N_A$  being Avogadro's number with a value of  $6.0222 \times 10^{23} \text{ mol}^{-1}$ ,  $[E]$ ,  $[S]$  are the concentrations of the enzyme and substrate molecules, respectively.  $R_E$ ,  $R_S$  are the effective radius (or gyration radius) of enzyme and substrate, respectively,  $T$  is the absolute temperature,  $k_B$  is Boltzmann constant with a value of  $1.3806 \times 10^{-23} \text{ JK}^{-1}$ ,  $\eta$  is the viscosity. The values of constants  $c_E$  and  $c_S$  obtained from the experiment reveal mainly the properties of the molecules etc. The pH, certain ions and certain chemicals will also affect the diffusion process and the value of these parameters [58,60], for example  $c_E$ ,  $c_S$ , or  $R^*$ .

Since gas constant  $R = k_B \cdot N_A$ ,  $k_B$  is the Boltzmann constant,

$$k_{\text{collision}} = (4RT/\eta) \cdot R^* \cdot [1/(c_E R_E) + 1/(c_S R_S)] \quad (4)$$

If  $R$  is used in unit of  $\text{J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ ,  $T$  in Kelvin,  $\eta$  in poise ( $P$ ,  $1 P = 0.1 \text{ kg} \cdot \text{m}^{-1} \cdot \text{s}^{-1}$ ),  $k_{\text{collision}}$  will have unit of  $\text{m}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ .

The encounter rate constant in aqueous solution is  $\sim 7.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$  ( $\text{mol}^{-1} \cdot \text{L} \cdot \text{s}^{-1}$ ) for two molecules with a molecular weight of 190 g/mol (approximately 1nm in size), with diffusion coefficient  $D_{1\text{nm}}$  of  $4.9 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ . For protein molecules with a molecular weight of 41 kDa (approximately 5 nm in size) in aqueous solution, the encounter rate constant is  $\sim 6.3 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ , and the diffusion coefficient  $D_{5\text{nm}}$  is  $8.3 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ . When the concentration of the molecules falls within milli molar (mM,  $10^{-3} \text{ M}$ ) range, the time it takes on average for an encounter is at about  $10^{-6} \text{ s}$  time scale; when the concentration of the molecules falls within micro molar ( $\mu\text{M}$ ,  $10^{-6} \text{ M}$ ) range, the time it takes for an encounter is at about  $10^{-4} \sim 10^{-3} \text{ s}$  time scale.

In the second circumstance, there is coulomb interaction (attraction or repulsion) between the reactants (for example between the enzyme and substrate) [61],

$$f(u) = (U/k_B T) / (e^{U/k_B T} - 1)$$

$$U = [e^2 / (4\pi\epsilon_0)] \cdot (Z_E Z_S / \epsilon_R R^*)$$

$Z_E$ ,  $Z_S$  are reactant charge numbers,  $e^2 / 4\pi\epsilon_0 = 2.307 \times 10^{-28} \text{ Jm}$ ,  $\epsilon_R$  is relative permittivity. If  $Z_E \cdot Z_S = 0$ , then  $f(u) = 1$ .

$$k_{\text{collision}} = 4\pi R^* N_A [k_B T / (c_E \pi \eta R_E) + k_B T / (c_S \pi \eta R_S)] \cdot f(u)$$

$$k_{\text{collision}} = (4RT/\eta) \cdot R^* \cdot [1/(c_E R_E) + 1/(c_S R_S)] \cdot f(u) \quad (5)$$

$$\text{Collision/encounter rate} = (4RT/\eta) \cdot R^* [1/(c_E R_E) + 1/(c_S R_S)] \cdot f(u) \cdot [E] [S]$$

For either circumstance,  $k_{\text{collision}}$  may be inversely proportional to the relative density of the system,  $k_{\text{collision}} \propto \rho_{\text{water}}/\rho$ ,  $\rho$  and  $\rho_{\text{water}}$  are the densities of the system and of pure water at the same specific condition, respectively.  $k_{\text{collision}}$  may be affected by the pressure of the solution system as well.

$k_{\text{dif}} = 1000 \cdot k_{\text{collision}} \cdot [S]$ ,  $[S]$  in  $\text{mol} \cdot \text{L}^{-1}$ ,  $k_{\text{collision}}$  in  $\text{m}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ ,  $k_{\text{dif}}$  will have unit of  $\text{s}^{-1}$ , collision/encounter rate/velocity can have unit of  $\text{M} \cdot \text{s}^{-1}$ . Similarly, another parameter  $k_{\text{difSub}} = 1000 \cdot k_{\text{collision}} \cdot [E]$  may be defined, which reveals the number of enzyme molecules one substrate molecule will possibly meet with on average in the solution system within one unit of time, in like  $\text{s}^{-1}$ . Therefore,  $k_{\text{collision}}$  is much more important a parameter than  $k_{\text{dif}}$ . This  $k_{\text{dif}}$  parameter is dependent on size and concentration of substrate, viscosity, temperature, pressure, density of the system, electrostatic attraction, electromagnetic effect, etc.

When the enzyme is nearly saturated by the SMS, the required effectual Brownian motion distance of the reactant is very short. The higher the concentration of the substrate molecules in the more inviscid or frictionless system, the shorter time the efficacious diffusion is required. The chances are there is substrate immediately available near the catalytic center [5,44,53], the larger the  $[S]$  and the slower the other steps, the greater the probability of this. If the enzyme catalyzed biochemical reaction is the conversion from an SMS to a small-molecular-weight product, the diffusion process will scarcely affect the catalytic rate or velocity significantly unless ① the combined process of binding, conformational change, chemical conversion and product-release steps is very fast, much faster than diffusion step, ② the reactant concentration is very low, ③ the system is very viscous, ④ there is repulsion between reactants.

## 7. Conformational Change and Structural Re-Organization/Rearrangement

Conformational change prepares both the enzyme and the substrate with correct geometry and electrostatics ready for substrate-to-product conversion, the enzyme and sometimes both the enzyme and the substrate perform conformational change. Conformational change can be induced by enzyme–substrate binding or can be a spontaneous process, the enzyme or substrate may sample a broad distribution of conformations and visit the chemical-conversion competent conformation with variable frequencies. For LBMS, the enzyme-LBMS complex frequently performs conformational change as an integrated unity [62]. Induced fit can have a very amplified scale and a different definition when it comes to macromolecular enzymes and macro-substrates. Conformational change is independent of and not coupled to the diffusion step.

Catalysis relevant conformational change can be classified into three different categories. First, the minor-scale conformational change of the active site that happens in parallel with the chemical step. Some conformational change and chemical steps may be coupled; for example, the bound substrate can be converted to product through the action (side chain vibration, rotation or flipping) of the residues at the catalytic center [63]. Local motion can be quick and fast, taking little time; this is a case where conformational change shows little observable additional constrain, aside from the general chemical-conversion step, on the catalytic rate, although the motion frequency of certain key active site residues may correlate with the chemical-conversion rate. For some enzymes or catalysis, both minor-scale conformational change and large-scale conformational change take place, and either can overlap to some extent to the substrate-to-product chemical conversion process. Second, the large-scale conformational change that is independent of and separable in time course from the substrate-to-product chemical conversion step [27,30,31,50]; this is the case when large-scale conformational change is correlated with molecular recognition or product release. Third, large-scale conformational change that happens in parallel or interspersed with the chemical-conversion step. In either case, large-scale conformational change may significantly affect the overall rate of catalysis.

For the three categories discussed above, the tiny-scale dynamics of the enzyme do not form an independent catalytic step. The large-scale conformational change does form an independent unique catalytic step. Large-scale conformational change accounts for the majority part of the dynamics of the enzyme, from both the time and space point of view; it takes the majority amount of time, and covers the majority scale of distance. The amount of time taken by the conformational change step mainly arises from large-scale conformational change. There are real cases where conformational change efficiency affects the catalytic rate/velocity of biochemical reactions [27,30,31]; this happens since large proportions of the enzyme are involved in large-scale conformational change. A couple of different factors can be given here about what may affect conformational change efficiency and rate/velocity.

Intrinsic factors of the enzyme or substrate that affect the conformational change efficiency. ①, the rigidity, stability and flexibility of the enzyme or substrate. For instance, thermal stable enzymes tend to have enhanced hydrogen bonding network, hydrophobic interaction and other weak interactions; these interactions collectively make the enzyme stable and contribute to the rigidity and reduced flexibility at ambient temperatures. The presence of linkers, hinges or long chains may contribute to the flexibility of the enzyme. ②, steric hindrance. Certain residues of the enzyme or substrate may hinder the conformational change through steric effect, thus reducing the conformational change efficiency. Enzyme–substrate interaction may be decelerated by steric frustration as well. ③, the conformational change efficiency of the enzyme–substrate complex as a whole can be affected by certain residues or certain factors, either accelerating or decelerating, while the conformational change of the free enzyme or free substrate is not affected. Modification or mutation of the enzyme will sometimes affect the conformational change efficiency through the second or the third mechanism.

Environmental factors that can affect the conformational change efficiency include: the presence or absence and concentration of certain chemicals, certain cofactors, ligands, modulators or certain regulatory molecules, pH and ion strength, etc. [64]. The pH of the solution system will influence the protonation state of both the enzyme and the substrate, and may thus affect electrostatic interaction and efficiency of conformational change. The enzyme may be hindered from or promoted to efficient conformational change because of protonation state change. The affinity between the enzyme and the substrate may be affected by the protonation state or pH as well.

The temperature, pressure, viscosity and density of the system can all affect conformational change efficiency [10,20,27,51,52,65–72]. The relationship between the conformational change coefficient and physical conditions of the solution system is proposed here.

$$k_{\text{conf}} = c_{\text{adjust1}} T p / (\sigma + \eta) + c_{\text{adjust2}} \quad (6)$$

$T$  is the absolute temperature measured in Kelvin,  $p$  is the pressure in pascal ( $1 \text{ Pa} = 1 \text{ kg} \cdot \text{m}^{-1} \cdot \text{s}^{-2}$ ),  $c_{\text{adjust1,2}}$  are adjustment constants,  $\eta$  is the viscosity in poise ( $P$ ,  $1 P = 0.1 \text{ kg} \cdot \text{m}^{-1} \cdot \text{s}^{-1}$ ),  $\sigma$  is another adjustment parameter with unit the same as viscosity.  $k_{\text{conf}}$  is measured in like  $\text{s}^{-1}$ .  $k_{\text{conf}}$  may be inversely proportional to the relative density of the system,  $k_{\text{conf}} \propto \rho_{\text{water}} / \rho$ ,  $\rho$  and  $\rho_{\text{water}}$  are the densities of the system and of pure water at the same specific condition, respectively. This equation supposes linear dependence of conformational change coefficient on the temperature, inverse of viscosity, and pressure of the system, probably not over the entire range but at least within certain windows (Figures S2 and S3). Catalytic kinetics, molecular dynamics simulation, structural biology including nuclear magnetic resonance (NMR) method, spectroscopy and single molecular technology etc. may be employed to study the conformational change step, and NMR has been extensively applied experimentally [10,27]. This equation supposes no melt of the enzyme or enzyme–substrate complex, especially no melt of the interdomain linker region if the conformational change happens between the two domains. Near the melting temperature, this function may not apply.  $c_{\text{adjust}}$  and  $k_{\text{conf}}$  of the conformational change

step at each of the pH, or at each of the type and concentration of certain chemicals, certain cofactors or modulators, etc., may be different.

Concerning how conformational change affects catalytic efficiency and rate, there can be three distinct pathways. First, conformational change affects the enzyme–substrate recognition/binding. Second, conformational change affects the substrate-to-product chemical conversion [22]; without the conformational change, the biophysical and biochemical environment of the active site will not be prepared ready for the successful chemical conversion. Third, conformational change affects the product release.

The long-range effect on catalysis is one of the revealing phenomena that conformational change step sometimes does affect catalytic rate seriously [43,73,74], and research has shown that for certain enzymes, conformational change accounts for up to 90% of the rate limits [50] at substrate saturation condition. Residues far away from the catalytic center show significant impact on both conformational change efficiency and catalytic rate, either accelerating or decelerating; those residues do not affect the catalytic center or the substrate-to-product chemical conversion step but affect the conformational change step [24,27]; conformational change-efficiency alteration is the primary cause of catalytic rate change [31].

For cases like enzyme-LBMS-ATP-compound1, enzyme and LBMS are major players of both diffusion and conformational change steps. What if enzyme and LBMS form a binary complex and then perform conformational change, while at the same time ATP and compound1 diffuse to bind to the binary complex? Is there overlap between the diffusion and conformational change steps? How can this be explained? Actually, letting this complicated process be simplified to these steps or similar may be a feasible and credible approximation. First, diffusion and encounter of enzyme and LBMS, then conformational change of enzyme-LBMS binary complex, then diffusion and encounter of enzyme-LBMS binary complex with ATP and compound1, the process of which is relatively swift.

## 8. Substrate-to-Product Chemical Conversion Step

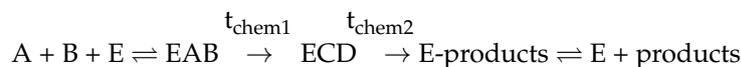
The classical kinetic theories actually illustrate the chemical conversion step explicitly. Equilibrium constant  $K_{eq}$  and Gibbs standard free energy change  $\Delta G^\circ$  describe the direction, favorability and the final state of the chemical conversion step, like the relationship between final concentrations of the product and the reactants ( $K_{eq}$ ), and the total energy released or absorbed during the reaction ( $\Delta G^\circ$ ). Equilibrium constant has a direct relationship with standard free energy change. From transition state theory and Arrhenius equation [1,75], the relationship between the activation energy  $\Delta G^\ddagger$  (or  $\Delta E_a$ ), temperature and the rate constant  $k_{Gibchem}$  of chemical steps are established [76–78],  $-\Delta G^\ddagger = RT \cdot \ln(k_{Gibchem} h / k_B T)$ . Two adjustment factors  $A_1$ ,  $A_2$  are introduced into the equation here to estimate a relationship between chemical conversion coefficient  $k_{chem}$  and temperature,

$$k_{chem} \approx A_1 (k_B T / h) e^{-A_2 \cdot \Delta G^\ddagger / RT} = A_1 (k_B T / h) e^{-A_2 (\Delta H^\ddagger - T \Delta S^\ddagger) / RT}$$

$R$  is the ideal gas constant ( $8.314 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ ),  $k_B$  is Boltzmann constant with a value of  $1.3806 \times 10^{-23} \text{ J} \cdot \text{K}^{-1}$ , and  $T$  is the absolute temperature.  $A_1$  of frequency factor  $A_1 k_B T / h$  and  $A_2$  need to be obtained experimentally.  $\Delta H$  and  $\Delta S$  are standard changes of enthalpies and entropies, respectively.  $k_{chem}$  may be proportional to a term of the pressure of the solution system,  $k_{chem} \propto p \cdot e^{-A_3/P}$ , and  $A_3$  is another adjustment factor. Why and how chemical steps are accelerated by the enzymes have been discussed before: enzyme lowers the activation energy. Actually, enzyme increases the possibility of bound substrate reaching the required state, thus speeding up the chemical step.

This Arrhenius equation works fine, particularly for the chemical-conversion step of a catalysis, but does not always work for the catalytic process as a whole. It is true within the chemical-conversion step that the sub-step which requires the highest activation energy is the rate limiting sub-step. If the whole catalytic cycle is concerned, certain steps like the physical or biophysical process can be rate limiting but can have nothing to do

with activation energy of chemical steps at all. Some catalysis or reactions do not follow equilibrium thermodynamics [34,79], but the master equations will still apply.



The overall rate of substrate-to-product chemical conversion step is mainly constrained by the rate limiting sub-step which requires the highest activation energy. The rate limiting sub-step of chemical process may follow the Arrhenius equation, then the whole chemical process roughly follows Arrhenius equation, and  $k_{\text{chem}}$  roughly equals to the rate limiting chemical sub-step  $k_{\text{chemi}}$ . If there are two rate-limiting chemical sub-steps,  $i$  and  $j$ , both may follow Arrhenius equation,

$$k_{\text{chemi}} = A_{i1} (k_B T/h) e^{-A_{i2} \cdot \Delta G_i^\ddagger / RT} = A_{i1} (k_B T/h) e^{-A_{i2} (\Delta H_i^\ddagger - T \Delta S_i^\ddagger) / (RT)}, \quad (7)$$

$$k_{\text{chemj}} = A_{j1} (k_B T/h) e^{-A_{j2} \cdot \Delta G_j^\ddagger / RT} = A_{j1} (k_B T/h) e^{-A_{j2} (\Delta H_j^\ddagger - T \Delta S_j^\ddagger) / (RT)}, \quad (8)$$

Consistent with the master equation, let

$$t_{\text{chem}} k_{\text{chem}} = 1, 1/k_{\text{chem}} = 1/k_{\text{chemi}} + 1/k_{\text{chemj}}, \quad (9)$$

then the 'Arrhenius equation' for the whole chemical-conversion step will be different from that of step  $i$  or  $j$ .

## 9. Product-Release Step

Sometimes, the product-release step can be the rate-limiting step [21,30,50]. On one occasion, the product-release process means huge conformational change, and the product-dispensing-conformational-change step can be time consuming. In another situation, the product may exhibit a strong affinity to the enzyme, resulting in slow release.

Can the product-release step largely overlap in time course with the substrate-binding step? Can product release happen at approximately the same time as substrate binds to the enzyme? It is possible, but not always. Sometimes, the presence of substrate facilitates the release of product, because the substrate has a higher affinity to the enzyme than the product. The reactant-conformational-change step and product-release step are not consecutive steps but separated by the chemical step. Will things change in essence if the start of a catalytic cycle is defined alternatively? Probably not. If another catalytic cycle is defined to start immediately after substrate is chemically converted to product, and all the events after this moment mean to prepare the enzyme ready for the next catalytic cycle, they are probably still two separate events, probably interrupted by the diffusion step in between.

If product release means large-scale conformational change, a function is proposed to describe the product-release kinetics.

$$k_{\text{prod}} = c_{\text{adjust-prod1}} T p / (\sigma + \eta) + c_{\text{adjust-prod2}} \quad (10)$$

Can this function be combined with that of the reactant-conformational-change step? Is it possible that the two functions merge into one, and different new values for factor  $c_{\text{adjust}}$  and  $\sigma$  are obtained after the combination? Maybe this is plausible in certain circumstances.

The rate or velocity of the product-release step depends on enzyme-product concentration [EP],  $V_{\text{prod}} = k_{\text{prod}} [\text{EP}]$ . Product dissociation kinetics need to be studied experimentally for further systematic analysis, especially for cases like rate-limiting product release caused by strong binding.

## 10. Explanations to Master Equations

The addable nature of the amount of time taken by each step of catalysis (Equation (1)), and the relationship between catalytic coefficient and time (Equations (2) and (3)) network

the constituent steps of catalysis into a unity. The flow of time is commonly regarded as unidirectional, irreversible and continuous; each of every enzyme molecule inevitably has to undergo each of every catalytic cycle through each of every step, unable to skip any one single step, as assumed by the first general presumption; both underlie the versatility of master equations. This is fundamentally distinct from previous theories, as catalytic coefficient  $k_{\text{step-}i}$  of any step- $i$  is absolutely not directly addable to each other here.

Classical theories on enzyme catalysis also utilize statistical concepts to describe the kinetics, but multiple enzyme molecules or possibilities are concerned at any given unit of time [5,44,53]. Whenever one writes  $k_1 ([E_t] - [ES]) [S] = [ES] (k_2 + k_{-1})$  or something similar, he/she consciously or unconsciously admits either of the following: ① Assuming  $k_2$  and  $k_{-1}$  as possibilities of one single ES pair going from ES to either directions. In this case  $k_2$  and  $k_{-1}$  are probability parameters, but in reality, the time a step takes is a real fixed value as long as the catalysis takes place, which may be measured by modern technologies. ② Assuming  $k_2$  and  $k_{-1}$  as amounts or number of molecules, some enzyme molecules ( $k_{-1}$ ) go from  $ES \rightarrow S + E$ , some others ( $k_2$ ) from  $ES \rightarrow E\text{-}p$ ; in this case  $k_2$  and  $k_{-1}$  are proportion, fraction or distribution parameters, this time they are something real but very difficult to relate to real concrete biophysical properties and hard to examine, as  $k_2$  and  $k_{-1}$  themselves are statistical values. Assuming either case, statistics are used to answer the proportion (probability or how many) going forward and backward. Statistics actually can be used alternatively where it is sampleable and statisticable. This writing utilizes each catalytic cycle as independent samples for statistics, rather than assuming the amount or chances of going in either direction by ensemble of enzyme molecules. In this way, this writing tries to elucidate catalytic kinetics of enzymes in aqueous solution at both molecular and general/macroscopic level and reveal the connection between them.

## 11. The Catalytic Cycle as a Whole

Kinetic experiment obtained parameters (like velocity, catalytic coefficient) are actually the averaged value, which indicate the regularities of the behaviors of bulk enzyme molecules, and this is linked here to the statistical analysis of singular enzyme catalytic behavior, which can be obtained for instance by single molecule techniques.

$$1/V_{\text{overall}} = 1/V_1 + 1/V_2 + \dots + 1/V_i \dots + 1/V_n \quad (11)$$

and equation for extent of catalysis per unit time is similar. And the functions in this study can be connected to and be applied to experimental study. A set of simultaneous equations can be used to achieve this. If all other steps are negligible, and if reactant conformational change and chemical conversion step can be combined,  $1/(k_{\text{conf}} \cdot [ES]) + 1/(k_{\text{chem}} \cdot [ES]) = 1/(k_{\text{conf-chem}} \cdot [ES]) = (t_{\text{conf}} + t_{\text{chem}})/[ES]$ , then

$$\begin{aligned} 1/k &\approx t_{\text{difu}} + t_{\text{conf}} + t_{\text{chem}} + t_{\text{prod}} \\ 1/V_{\text{overall}} &\approx 1/(k_{\text{collision}} \cdot [E] \cdot [S]) + (t_{\text{conf}} + t_{\text{chem}})/[ES] + t_{\text{prod}}/[EP] \\ 1/t_{\text{difu}} &= k_{\text{collision}} \cdot [S] \end{aligned} \quad (12)$$

The overall catalytic rate or velocity  $V_{\text{overall}}$  and catalytic coefficient  $k$  of the catalysis can be examined and obtained experimentally. A lot of parameters need to be acquired from the experiment to resolve these functions. The situation may be further simplified according to three different scenarios. First, the diffusion step is rate limiting, and all three steps (conformational change, chemical conversion and product release) combined are not rate limiting, then  $k_{\text{conf-chem-prod}}$  (or  $t_{\text{conf}} + t_{\text{chem}} + t_{\text{prod}}$ ) probably need not to be considered;  $[ES]$  is changing as  $[S]$  decreases. Then,  $V_{\text{overall}} \approx k_{\text{collision}} \cdot ([E] \cdot [S])$ , and Equations (4) or (5) may be used together to estimate the ratio of unproductive collision, which is

$$\begin{aligned} &1 - [V_{\text{difu}}/([E] \cdot [S])]/[(4RT/\eta) \cdot R^* \cdot [1/(c_E R_E) + 1/(c_S R_S)] \cdot f(u)] \\ &= 1 - [(k_{\text{difu}}/k) \cdot V_{\text{overall}}/([E] \cdot [S])]/[(4RT/\eta) \cdot R^* \cdot [1/(c_E R_E) + 1/(c_S R_S)] \cdot f(u)] \\ &\approx 1 - [V_{\text{overall}}/([E] \cdot [S])]/[(4RT/\eta) \cdot R^* \cdot [1/(c_E R_E) + 1/(c_S R_S)] \cdot f(u)], \end{aligned} \quad (13)$$

if all other steps are negligible.

Second, the diffusion step is fast, the three steps combined is rate limiting.  $[ES]$  is virtually constant, so that steady-state approximation can be applied. Then  $1/k \approx 1/k_{\text{conf-chem-prod}} = t_{\text{conf}} + t_{\text{chem}} + t_{\text{prod}}$ ,  $1/V_{\text{overall}} \approx (t_{\text{conf}} + t_{\text{chem}})/[ES] + t_{\text{prod}}/[EP]$ , and actual  $k_{\text{conf-chem-prod}}$  is slightly larger than the experimentally obtained  $k_{\text{exp}}$ . The values of  $t_{\text{conf}}$ ,  $t_{\text{chem}}$ ,  $t_{\text{prod}}$  need to be further examined to see which one or ones dominate. Third, both diffusion and the three steps combined are rate limiting.

$$1/k \approx t_{\text{difu}} + t_{\text{conf-chem}} + t_{\text{prod}}$$

$$1/V_{\text{overall}} \approx 1/(k_{\text{collision}} \cdot [E] \cdot [S]) + t_{\text{conf-chem}}/[ES] + t_{\text{prod}}/[EP]$$

If  $t_{\text{conf-chem}}/[ES] + t_{\text{prod}}/[EP]$  can be approximated by  $t_{\text{conf-chem-prod}}/[ES]$ , like overall rate or velocity of three steps combined (conformational change, chemical conversion and product release) approximately depends on the concentration of enzyme–substrate complex,  $V_{\text{conf-chem-prod}} \approx k_{\text{conf-chem-prod}} \cdot [ES]$  or  $V_{\text{conf-chem-prod}} \approx k_{\text{conf-chem-prod}} \cdot [ES_1S_2S_3 \dots]$ , then,  $1/V_{\text{overall}} \approx 1/(k_{\text{collision}} \cdot [E] \cdot [S]) + t_{\text{conf-chem-prod}}/[ES]$ .

To study an enzyme's kinetics in detail, the catalytic condition may be deliberately set so that the throughput capacity of each step can be resolved. For instance, by working at very low enzyme concentrations and substrate saturation conditions may be arranged so that diffusion takes negligible time; enzyme and substrate may be designed to temporally absorb at two ends of the solution system by using for example electrostatics/charges so that diffusion can be investigated in detail; enzyme and substrate may be premixed at appropriate stoichiometry and then catalysis initiated at a certain point to make chemical-conversion and product-release step rate limiting, etc. The contribution of each step  $t_{\text{difu}}$ ,  $t_{\text{conf}}$ ,  $t_{\text{chem}}$  and  $t_{\text{prod}}$  may be further analyzed with the aid of biophysical and biochemical technologies. In this way, the set of simultaneous equations listed above may be resolved for the specific catalysis or enzyme.

Binding energy contributes to reaction specificity and catalysis, this is the classical expression about the relationship between binding energy and catalysis. But previously, the relationship between binding efficiency and catalytic efficiency (or catalytic rate) is not clear. Now, correlation between enzyme-macro-substrate recognition/binding efficiency, conformational change efficiency and catalytic rate, between chemical-conversion efficiency and catalytic rate, and between product-release efficiency and catalytic rate, are discussed and linked to parameters obtained from kinetic, biophysical and biochemical experiments. Efficient binding, by itself, definitely contributes to catalysis by increasing catalytic cycle numbers per unit time [5,44,53,80]. The binding energy can reduce the activation energy of the chemical-conversion step, thus accelerating the chemical step, but strong binding may slow down the product release or enzyme-product-complex dissociation. Therefore, affinity, binding energy or dissociation constants obtained from biophysical experiments do not necessarily correlate directly to the catalytic velocity or catalytic coefficient or other parameters obtained from kinetic experiment. Here an atypical example is discussed. For LBMS involved multiple reactant catalysis, the binding energy between enzyme and LBMS does not necessarily contribute to the velocity of the catalysis directly, even if it accounts for the majority of the binding energy. Binding energy is not always the driving force and sometimes not the only driving force for catalysis. For instance, for nucleotide triphosphate (NTP) involved catalysis, usually the catalysis comes to a halt in the absence of NTP. Reaction coupling or the step-by-step release of covalent bond energy as one sequential reaction can explain the driving effect of NTPs. A detailed mechanism-based explanation of the relationship between binding efficiency, binding energy and catalytic rate requires further investigation.

Huge enzyme machinery catalyzed complex biosynthesis includes multiple rounds of conformational change, chemical conversion, etc. to manufacture a single macromolecular product. For these complex biochemical reactions, the chemical-conversion process, conformational change and other steps may be interspersed with one another. Biosynthesis

are very sophisticated long-lasting processes and can repeat to generate certain amounts of macromolecular copies [81–87]. The catalytic step of these complex catalysis may be defined differently from above, and master equations still can be applied.

## 12. Summary and Perspectives

This writing tries to explain catalytic kinetics from a molecular level point of view. This theoretical writing proposes the master equations based on the sequential events along the time axis of catalysis, and tries to link the kinetic experimental results to microscopic catalytic steps. Inspected microscopic molecular events from this detailed kinetic study will provide fresh insight into the catalytic mechanism of enzymes. The relationship between catalytic rate, catalytic coefficient and substrate concentration, features of the reactant, biophysical, biochemical conditions of the system etc. can explain various experimental phenomena in general. This writing can be applied to comprehensively study the catalytic mechanism and catalytic kinetics of enzymes of interest. From this discussion, a systematic and much more balanced analysis of the catalytic process is possible. For instance, the catalytic rate is affected by temperature; this is not only because temperature affects the activation of the chemical-conversion step, but also because temperature affects the biophysical steps of each catalytic cycle as well.

With the advancement of science and technology, especially with the development of biophysics and single molecular manipulation and detection techniques [88–91], study in detail and in depth of the catalytic behavior of singular enzymes will become feasible, which will revolutionize the current understanding on the catalytic mechanism of enzymes. Then, it will be possible for enzyme kinetic functions, parameters, equations and descriptions to suit the situation, experimental results to be interpreted faithfully to fully agree with molecular events, and what's really happening in the process of catalysis to be genuinely and accurately revealed. Extensive further experimental research is required to combine this writing, classical kinetic theories and experimentally obtained single molecular actual behavior [92].

Now it is clear that enzyme catalytic efficiency and catalytic rate/velocity can be affected by so many factors at so many steps, features of catalytic process can be fine-tuned, and a lot of different strategies or approaches can be utilized for enzyme engineering, catalysis engineering, disease control and prevention, drug discovery [93,94], signaling pathway manipulation, metabolic pathway modulation, artificial molecule design or bioreactor design and so on. For instance, the  $c_{\text{adjust}}$  factor ( $c_{\text{adjust}1,2}$ ,  $c_{\text{adjust-prod}1,2}$ ) of the conformational change process and factor  $A$  ( $A_{1,2}$ ) of the chemical-conversion step, which depict the intrinsic properties of reactant (enzyme, substrate or ES complex) at specific chemical conditions, may be used as the parameters to quantitatively evaluate the optimization effort in enzyme engineering or directed evolution.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/2227-9717/9/2/271/s1>, Figure S1: Michaelis–Menten curve, Figure S2: Re-plot of published experimentally obtained correlation between temperature of the system and the turnover number ( $k_{\text{cat}}$ ), Figure S3: Re-plot of published experimentally obtained correlation between pressure of the system and the turnover number ( $k_{\text{cat}}$ ).

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**Data Availability Statement:** The data presented in this study are available in article or Supplementary Material here.

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